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## RESEARCH PROGRESS

One goal this research project has been to assess the dependence of breast cancer cell survival on the PI3 kinase/Akt signaling pathway. This signaling pathway is hypothesized to be critical to breast cancer survival especially under growth-limiting conditions or when chemotherapeutics are present. Using a series of six breast cancer cell lines and a phenotypically normal, but immortal, breast line I first determined the baseline level of phosphorylated Akt as an indicator of basal PI3 kinase signaling. Phosphorylated ERK1/2 was also measured to determine if basal survival signaling through the MEK1/2→ERK1/2 pathway was occurring. While all of the breast cancer cell lines expressed basal levels of phosphorylated ERK1/2, phosphorylated Akt was only found in a fraction of the cell lines. The removal of exogenous growth factors by culture in serum-free media did not significantly change the detected level of phosphorylated Akt and ERK1/2 expressed by the cells, suggesting that PI3 kinase→Akt and MEK1/2→ERK1/2 signaling occurs in the absence of exogenous growth factors. Pharmacological inhibitors of these key survival signaling pathways were tested for their affects on breast cancer cell survival under both serum-free and serum-containing conditions. Interestingly, the results to date suggest that most breast cancer cell lines rely more heavily on the MEK1/2→ERK1/2 signaling pathway than on the PI3-kinase→Akt pathway for survival.

Several studies have established the importance of survival signaling through the PI3 kinase→Akt pathway during acute stress, however very little is known about the role of this pathway during prolonged stress. Understanding how cancer cells survive under nutrient-limited conditions and periods of extended stress, such as occur during chemotherapy, could provide novel targets for improving current therapies. In this study cells were deprived of serum for several days and assayed for survival/viability and growth as well as phosphorylated Akt and ERK1/2 levels. Important survival signals originate from outside the cell. Growth factors, through interactions with their receptors, have been found to modulate both growth and survival signaling pathways. By eliminating serum, all exogenous growth factors were removed from the extracellular environment. It was expected that lack of serum would limit growth. Survival and growth were measured by quantitating the number of viable cells in a culture over time. Viability was determined by exclusion of trypan blue dye. I also looked at the effect of serum-free media on the proliferative potential of breast cancer cells. To test whether the viable cells, which remained in each culture after various days of serum-free media, retained the ability to proliferate, I developed a simple assay to measure proliferative potential. Following exposure to adverse conditions, cells were re-plated in serum-containing media. Growth was assessed by measuring the increase in DNA content per well over a series of days. This type of assay was also used to determine the effects of various chemotherapeutics on proliferative potential. In addition to providing information about the cytotoxic or cytostatic effect of a given treatment, the practice of measuring viability and proliferative potential provides critical information with regard to the ability of the remaining cells to repopulate under less-stressful conditions. Together these measurements may more accurately model what occurs *in vivo* following treatment.

*Effect of serum deprivation on cell survival:* Survival time under serum-free conditions varies among the cell lines. Several of the breast cancer cell lines, as well as the phenotypically normal but immortalized MCF-10A line, contained a significant portion (>40% of the number initially plated) of viable cells after more than 20 days in serum-free media. This demonstrates that the absence of exogenous growth factors does not immediately induce a significant level of death in these lines. It is possible that these cells have altered signaling pathways which obviate the need for growth factors or that they secrete enough growth factors to keep the majority of the population alive. Two of the breast cancer cell lines, MDA-MB-468 and ZR-75-1, actually grew significantly in serum-free media, indicating they receive replicative signals from sources other than exogenous growth factors. Two other lines, SKBr3 and T47D, doubled once and then began to lose viability rapidly. The remaining lines did not appear to grow in serum-free media and essentially kept a constant number of viable cells for several days (>7 days). In most cases, the viable cells retained proliferative potential. Survival time in suspension, which limits survival signals emanating from the cell matrix, also varied among the cell lines and was less than the survival time for the same cells attached to plastic.

*Effect of serum deprivation on the expression of phosphorylated ERK1/2 and Akt:* Viability and proliferative potential of the seven cell lines in serum-depleted media correlated with the presence of phosphorylated ERK1/2 expression. Phosphorylated ERK1/2 levels were not significantly changed 2 days after serum removal and were still detectable while viable cells were present. In the ZR-75-1 cell line only, viability and proliferative potential correlated with the presence of both phosphorylated ERK1/2 and phosphorylated Akt. Survival of the other cell lines did not correlate with the presence of phosphorylated Akt. While phosphophorylated Akt was present in some of the cultures that retained viability following several days in serum deprived media, there was no case in which only phosphophorylated Akt in the absence of phosphophorylated ERK correlated with survival. These data suggest that phosphophorylated ERK, rather than Akt, correlates with breast cancer survival under serum-free conditions.

I next tested the effect of pharmacologic inhibitors of MEK1/2 and PI3-kinase signaling on breast cancer cell survival. U0126 and PD98059 were used to inhibit MEK1/2. LY294002 was used to inhibit PI3-kinase signaling. At a dose of 20 $\mu$ M these inhibitors effectively blocked induction of phosphorylated ERK1/2 or Akt by EGF or IGF-1. Since these signaling pathways are linked to both growth and survival, I measured the effect of the inhibitors on viability, proliferation, cell-cycle status, and proliferative potential. The phosphorylation status of downstream targets (mainly phosphorylated ERK1/2 and Akt) was also measured in order to determine the effectiveness of the inhibitor on signaling. These studies were conducted in both serum-free and serum-containing conditions.

*Effect of U0126 on cell survival:* U0126 and LY294002 each inhibited the proliferation of all seven breast cell lines. Cell cycle data show that U0126 induces an accumulation of cells in the G0/G1-phases and loss of cells in the S-phase of the cell cycle in many, but not all, of the cell lines. Interestingly, the two cell lines that did not display a G0/G1 arrest were the phenotypically normal MCF-10A cells and the SKBr3 breast cancer

cell line. The SKBr3 cells actually accumulated a sub-G1 population indicative of apoptotic death. These cells also displayed an increase in PARP cleavage over time, suggesting caspase activation. Several of the U0126-arrested cell lines had an early increase in the protein levels of p27, a cell cycle inhibitor, and a decrease in cyclin D1 levels consistent with a G1 arrest. Under serum-deprived conditions, U0126 treatment eventually resulted in a complete loss of viability and proliferative potential in each cell line. There was a slight differential in response time between the cell lines, but no breast cancer cell line contained any viable cells after 4-5 days of treatment. Notably, the MCF-10A cells, while growth inhibited, maintained a viable subpopulation for up to 7 days. The difference in survival time under serum-containing vs. serum-free conditions plus U0126 was negligible for most cell lines. An exception to this finding was the MDA-MB-468 and MCF-10A lines, which survived for several (>10) more days when serum was present. MDA-MB-468 and MCF-10A cells may utilize a serum-requiring survival signaling pathway not operating in the other cell lines.

The effect of U0126 is initially reversible. Cells treated with 20 $\mu$ M U0126 in serum-free media for one day, proliferated when placed in complete media without the drug. However, after two days of treatment all but one of the breast cancer cell lines lost this proliferative potential. The MCF-7 line maintained the ability to proliferate after two days of U0126 treatment, but not after three days. MCF-10A cells retained proliferative potential for the longest period, three days. These results demonstrate that U0126 treatment for 2-3 days is sufficient to prevent the re-growth of each breast cancer cell line, despite the presence of viable cells. The results suggest that a single treatment of U0126 is capable of inducing an irreversible growth arrest and eventual death. Serum extended the proliferative potential of some of the cell lines. MDA-MB-231 and ZR-75-1 cell lines retained cells with proliferative potential for 2 days longer when serum was present. The most dramatic effects of serum were on the MDA-MB-468 and MCF-10A cell lines. Serum extended the proliferative potential of these lines by at least 10 days.

*Effect of U0126 on phosphorylated ERK1/2 levels:* U0126 effectively reduced phosphorylated ERK1/2 levels in each cell line. A single dose of 20 $\mu$ M U0126 depleted both basal and EGF or IGF-1 induced phosphorylated ERK1/2. The extent of repression in MEK1/2 $\rightarrow$ ERK1/2 signaling varied among the cell lines. Specifically, the MDA-MB-468 cell line displayed reduced but still detectable levels of phosphorylated ERK1/2 following U0126 treatment. The other cell lines had levels of phosphorylated ERK1/2 that were barely detectable after treatment. In some cell lines a wave of ERK1/2 activity was noted after 2-3 of treatment, but the level of phosphorylated ERK1/2 was always less than that of the time-matched control.

The onset of U0126-associated death correlated with the loss of Bcl-2 and Bcl-X<sub>L</sub> protein levels in 5 of the 6 breast cancer cell lines. Bcl-2 and Bcl-X<sub>L</sub> are antiapoptotic proteins known to regulate apoptosis induced by a variety of cytotoxic insults. There is some evidence that these proteins may be regulated by MEK1/2 $\rightarrow$ ERK1/2 signaling. At this point, I have no data to suggest the loss in Bcl-2 or Bcl-X<sub>L</sub> contributes directly to U0126-induced death. Further studies will be done to determine if loss of these proteins is a causative factor in the death seen with U0126 treatment.

*Effect of LY294002 on cell survival:* Unlike U0126, LY294002 treatment did not induce death in every cell line. LY294002 did, however, arrest the growth of all of the cell lines, even those with nearly undetectable levels of basal phosphorylated Akt. As noted earlier, survival of the ZR-75-1 cell line correlated with the presence of both phosphorylated ERK1/2 and Akt. The survival of this cell line in particular was affected by LY294002 treatment. There was a very small reduction in the number of viable ZR-75-1 cells after 3 days of LY294002 treatment, but a complete loss in viability after 9 days. The cell line did not lose complete viability as quickly as it did with the MEK1/2 inhibitor, but the ability of U0126 and LY294002 to separately eliminate ZR-75-1 cell survival suggests that both the MEK1/2→ERK1/2 and PI3-kinase→signaling pathways are required for their survival. In contrast, since the majority of the breast cancer cell lines survived LY294002 treatment, PI3 kinase→Akt signaling may be more critical for cell growth than survival in these lines.

*Effect of LY294002 on phosphorylated Akt levels:* As noted earlier, a single dose of LY294002 (20μM) effectively blocked EGF or IGF-1 induced phosphorylation of Akt. However, LY294002 did not have a dramatic effect on basal phosphorylated Akt levels. Often, little if any, reduction in phosphorylated Akt was noted after 24 hours of exposure. Interestingly, I found that LY294002 treatment of ZR-75-1 cells resulted in a reduction of phosphorylated ERK1/2 levels. These results strengthen the possibility that there may be cross-talk between the PI3 kinase→Akt and MEK1/2→ERK1/2 pathways. The results also add support to the hypothesis that the MEK1/2→ERK1/2 pathway is critical to breast cancer cell survival.

*Effect of U0126 plus LY294002 on survival:* The combination of U0126 and LY294002 was tested for its effect on viability. Combining the inhibitors resulted in enhanced death in the MDA-MB-468 and ZR-75-1 cell lines only. The other cell lines displayed no increase in death compared to U0126 alone. Notably, in each of these cell lines LY294002 treatment alone reduced phosphorylated ERK1/2 levels. Since phosphorylated ERK1/2 levels were reduced, but not completely eliminated at each time point after U0126 treatment, it is possible that the combination is more effective because it completely limits ERK1/2 signaling. The mechanism of this enhancement will be studied in more detail over the next year.

The results described above have complete many of the objectives of Tasks 1 and 2 and are being prepared for publication. As noted in my first report, the preliminary findings shifted the focus of Task 2 to put more emphasis on the effectors of the MEK1/2→ERK1/2 signaling pathway. This work is in progress. Since cells treated with U0126 can be rescued if the compound is removed early enough, I've been interested in understanding what occurs at the critical point when cells can no longer be rescued.

The aims of Task 3 are currently being addressed. Some experiments have been conducted to test the efficacy of combining U0126 or LY294002 with currently used chemotherapeutics. Using the cell culture model, I have tested the effects of adriamycin, taxol, 5-fluoruracil, navelbine, SN-38 (active form of irinotecan) or herceptin combined

with U0126 and/or LY294002. Studies involving blockade of the signaling pathways either before or after the chemotherapeutic treatment, are also being done.

*Effect of U0126 plus taxol on survival:* A slight increase in nuclear condensation has been found in a majority of the breast cell lines when U0126 is combined with taxol (100nM) or navelbine (1µM). The other cell lines actually displayed a decrease in nuclear condensation, suggesting protection. Most of the cell lines show no increase in nuclear condensation after U0126 alone for 16 hours. In addition to measuring apoptosis I have focused on the effects of the drug combinations on viability, proliferative potential and protein expression. Lower doses of taxol (1-10nM) for 16 hours did not prevent re-growth of the breast cancer cell lines. When combined with U0126, re-growth was limited in only a couple of the cell lines. In many cell lines U0126 combined with low dose taxol actually led to re-growth, indicating protection. Cell cycle analysis of some of these cell lines showed that taxol induces a G2/M arrest, but when combined with U0126 a G0/G1 population is present. Future studies will continue the analysis of the effects of combining the signaling inhibitors and chemotherapeutic drugs.

### TRAINING

Over the past year I have presented my research at biweekly lab group meetings, attended weekly seminars in the Department of Pharmacology and Toxicology, monthly Molecular Therapeutics meetings and seminars in the Norris Cotton Cancer Center. I presented my findings at a poster session of the AACR annual meeting in March 2001. Currently, manuscripts are being prepared to describe these findings.

## KEY RESEARCH ACCOMPLISHMENTS

Task 1: Assess the dependence of breast cancer cell survival on PI3 kinase/Akt pathway

A. Determine the effect of culture conditions on survival of several breast cancer cell lines

- Survival time of breast lines in serum-depleted media is varied.
- Two of the seven cell lines show appreciable growth (>2 population doublings) in serum-depleted media.
- Survival time of breast lines in suspension is varied.
- The MEK1/2 inhibitor, U0126 (20 $\mu$ M), arrests cell growth and reduces survival time in both serum-containing and serum-depleted media in all (6) of the breast cancer cell lines tested.
- The PI3 kinase inhibitor, LY294002 (20 $\mu$ M), arrests cell growth but has little to no affect on survival time of 4 of the breast cancer cell lines in serum-containing and serum-depleted media.
- LY294002, reduces survival time of the MCF-7 and ZR-75-1 breast cancer cell lines in serum-containing and serum-depleted media.
- Despite retaining viable cells for 4-5 days, all of the breast cancer cell lines treated with U0126 in serum-free media lose proliferative potential (ability to proliferate in complete media without drug) after 2-3 days.
- Serum does not significantly extend the survival time of most (5/6) of the breast cancer cell lines treated with U0126, but it does dramatically enhance the survival of MDA-MD-468 cells and the 'normal' breast line, MCF-10A.
- Serum extends the time in which three of the six breast cancer cell lines retain proliferative potential following treatment with U0126.
- Proliferative potential of every cell line was retained for at least 6 days after treatment with LY294002.
- Serum extends the time in which 4 of the breast cancer cell lines retain proliferative potential following LY294002.

B. Confirm the dependence of survival on the PI3 kinase/Akt and MEK/ERK pathways

- All of the breast cancer cell lines express measurable levels of phosphorylated ERK1/2.
- Phosphorylated Akt levels vary among the breast cancer cell lines; one line, MDA-MB-231, has no detectable phosphorylated Akt.
- MCF-10A cells, which are phenotypically normal, express low levels of phosphorylated ERK1/2 and no detectable phosphorylated Akt.
- Exposure to serum-free media for 48h does not significantly change phosphorylated Akt or ERK1/2 levels.
- Under prolonged serum-free conditions, all cultures that retain a viable sub-population and proliferative potential express phosphorylated ERK1/2.
- Under prolonged serum-free conditions, some of the cultures that retain a viable sub-population and proliferative potential expressed phosphorylated Akt.
- Viability and proliferative potential of ZR-75-1 cells correlates with the presence of both phosphorylated ERK1/2 and Akt.

- In no case does viability and proliferative potential correlate with the presence of phosphorylated Akt alone.
- Treatment with EGF or IGF-1 elevates phosphorylated ERK1/2 and phosphorylated Akt levels under serum-deprived and serum-containing conditions.
- U0126 blocks the induction of phosphorylated ERK1/2 by EGF or IGF-1.
- LY294002 blocks the induction of phosphorylated Akt by EGF or IGF-1.
- U0126 reduces the basal level of phosphorylated ERK1/2 in every cell line under both serum-deprived and serum-containing conditions.
- LY294002 reduces the basal level of phosphorylated Akt in only a fraction of the cell lines.

Task 2: Define downstream effectors of PI3 kinase/Akt and MEK/ERK pathways

- In 5 of the 6 breast cancer cell lines U0126 treatment (30h) leads to an accumulation of cells in the G0/G1 stage and reduction in the number of cells in the S stage of the cell cycle.
- U0126 treatment leads to an early increase in p27 levels in several of the cell lines.
- M30 levels, a measure of caspase activity, increase after U0126 treatment (26h) in 5 of the 6 breast cancer cell lines.
- After U0126 treatment, some cell lines have an increase in PARP cleavage over time (a measure of caspase activity).
- Five breast cancer cell lines display a reduction in the level of anti-apoptotic Bcl-2 or Bcl-X<sub>L</sub> proteins at times that correlate with U0126-induced death.
- Some, but not all, of the cell lines have elevated levels of phosphorylated JNK following U0126 treatment.

Task 3: Determine how selected anticancer agents deregulate survival pathways to induce apoptosis

- Survival curves of each line exposed to adriamycin, taxol, 5-fluorouracil, SN-38 and navelbine  $\pm$  U0126 or LY294002.
- Taxol (100nM) plus U0126 (16h) results in slightly increased levels of nuclear condensation in 5 of the 7 breast cell lines.
- Taxol (100nM) plus U0126 (16h) results in slightly lower levels of nuclear condensation in 2 of the 7 breast cell lines.
- Navelbine (1 $\mu$ M) plus U0126 (16h) results in slightly higher levels of nuclear condensation in 5 of the 7 breast cell lines.
- Naveline (1 $\mu$ M) plus U0126 (16h) results in slightly lower levels of nuclear condensation in 2 of the 7 breast cell lines.
- MDA-MB-468 and SKBr3 cells treated with 10nM taxol (24h) arrest in the G2/M phase of the cell cycle and show no re-growth when placed in complete media.
- MDA-MB-468 cells treated with 10nM taxol and U0126 (24h) have cells in the G0/G1 and G2/M phases of the cell cycle and are able to re-grow when placed in complete media.
- SKBr3 cells treated with 10nM taxol and U0126(24h) arrest in the G2/M phase of the cell cycle and show no re-growth in complete media.